Review of Group H proposal, "GC-MS Determination...."

Overall Evaluation: Excellent (Range includes Outstanding, Excellent, Very Good, Good, Fair, and Poor); Percentile Rank = 10% (top 10%).

This is a very creative proposal addressing the possibility of meat containing steroids/hormones. The scope of the project is large, maybe even too large, and will be demanding on the principal investigators (PIs). The experimental detail is very high, but the rationale behind the methods is not at all clear (totally lacking in most instances, e.g. derivatization of samples for GC). This is a solid proposal, but it could be improved by addressing the following areas.

1. The main hypothesis of the proposal is not very clear. Is it the point of the proposal to determine whether there is any meat in the study group of meats which contains steroids/hormones?

2. The proposal would improve if the PIs were to use headings throughout the proposal that allowed the various sections to be more readily identifiable.

3. The PIs should reorganize the introduction so that the idea of implantable devices is more clear. We hear of the devices early on but do not see what they are until page 2 (bottom).

4. There is no timeline for milestones for the grant period.

5. It was good of the PIs to provide the naturally occurring amounts of compounds in certain foods, but they neglected to describe the action of the natural and synthetic materials on the human body. Is there a risk from consuming any of these compounds? If so, at what levels?

6. A flow chart describing how the samples are treated would have been very beneficial.

7. Benefits and Future Work section is far too brief.

8. There is no description of data handling/treatment.

Grade = 90%

H-2

GC-MS Determination of Hormone Growth Promoters in Store Bought Beef Using Solid Phase Extraction for Separation and Purification

Group A

spell check

Abstract:

Natural and synthetic hormones have long been used by farmers to promote growth in live-stock. Although there is no conclusive scientific evidence that these hormones pose a health threat, the European market has completely banned their use as growth promoters. While the US allows the use of growth promoters, legal limits have been set. Some US companies market their products as being hormone free. Using GC-MS analysis of samples purified by solid-phase extraction cartridges we will determine the levels of natural and synthetic hormones in store bought beef.

Project Description:

The interest in this project began due to a recent news article on the Internet discussing the ban of imported meats from the United States to Switzerland. The shipment was found to contain diethylstilbestrol (DES). DES is an illegal steroid which has been banned for use in the United States. However, according to the article the FDA has not tested for use of DES in several years due to the fact that DES is not currently on the market. In the light of the situation the FDA stated they would again start testing for DES, but this would take some time to implement. The contaminated shipments were from two different companies in the United States, Bruss which is based in Chicago and Farmland National based in Liberal, Kansas.

DES was the first hormonal growth stimulant used in cattle.[1] Approval of DES was based on the level of 3 ppb. This level was considered extremely low at the time, especially when compared to the level of the doses of DES that were being used for the prevention of miscarriage in humans (later found not to work) and as a contraceptive. DES was discontinued in 1979, after being used for 25 years; however, it was not due to any safety problems associated with its use in cattle.[1] DES is a known carcinogen.

The European Union (EU) bans the use of steroids as growth promoters in domestic and imported beef. This ban is of great political importance due to the fact that US farmers routinely use hormones to make cattle grow faster and produce more milk. The World Trade Organization has been trying to decide if there is any scientific evidence that the use of hormones in cattle might endanger human health, and the European Economic Community (EEC) Scientific Committee has met to study the safety of growth promotants. According to the chairman, Dr. Eric Lamming, of Great Britian, the "the EU ban was instituted, in spite of scientific evidence, as a political solution to problems of food surpluses generated by the EU's Common Agriculture Policy (CAP)"[1]

The World Trade Organization Agreement, signed in 1994, included *The Agreement on Sanitary and Phytosanitary Measures*. Under this agreement, "countries will only be permitted to impose those requirements which are needed to protect health and which are based on scientific principles."[1] Relevant international standards such as those adopted by Codex Alimentarius were agreed upon by those member governments of the WTO. In 1995, member nations of the WTO agreed that the Codex Commission's

decision-making process is based on science despite the EU's opposition. This agreement implemented the maximum residue levels (MRLs) for the synthetic hormones and confirmed that there was no need to establish MRLs for natural hormones. The US then filed a complaint against the EU's ban on implanted beef with the WTO. The WTO ruled in favor the US on the basis of three areas: "unilateral import bans require scientific proof of health risks; EU legislation of hormone use is inconsistent with less strict feed additive rules;and Codex has set MRLs for the use of growth promotants which the EU should recognize."[1]

The European Commission is trying to substantiate its ban on importation of beef containing growth hormones by conforming with the provisions outlined in the WTO Sanitary and Phytosanitary Agreement. Since February, the European Comission has been ready to offer compensation to its partners. "On the 26th of July the WTO authorized the United States to suspend tariff concessions covering EU trade up to an amount of $116.8 million."[2]

According to the European Commission's website dated May 3, 1999 growth hormones in meat pose risk to consumers, and they believe there are different levels of evidence.[2] These findings are from the Scientific Committee for Veterinary Measures relating to Public Health. "For all six hormones endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects could be envisaged, but the available data does not enable a quantitative estimate of the risk. Even exposure to small levels of residues in meat and meat products carries risks and no threshold levels can be established for any of the six substances, stress the experts."[2]

The Committee also argues that the risk factors are increased by the fact that regulatory controls over residues of hormones of beef placed on the market in the United States are deficient. They argue several areas of deficiency which include:[2]

- Approved hormonal growth promoters are readily available over the counter without a veterinary prescription or supervision.
- Implantation is restricted to the ear but it is possible to make the injection anywhere posing an increased risk to the consumer if this site enters the food chain.
- The US does not perform regular meat inspection regulations for regular checks of animal carcasses for misplaced implants, even though in 1986 the US inspection service reported widespread misuse of hormone implants.
- The insufficient enforcement of lack of residue control programs for the probability of detecting misplaced implants, off-label uses, natural hormones, and the use of black-market substances. S/ ?

The Scientific Committee on Vetrinary Measures relating to Public Health therefore concluded that the risk concerning misuse would result in even higher tissue residue.

Hormones are used to increase the rate of weight gain and improve the efficiency of feed use by livestock; therefore, livestock gain weight faster on less feed and can be slaughtered faster with lower production costs. Hormones have been used since 1954.[1] Hormones also reduce the amount of fat in meat. It is estimated that 70-90 percent of feedlot cattle are implanted.[3] The primary compounds used in implants are androgens and estrogens but progestins can also be found. Implanted refers to the process by which hormones are delivered. A hormone pellet is injected under the skin on the back side of the ear. Hormones are implanted four to seven times during an animal's life.[4]

You should have discussed this earlier!

Six hormones are approved for use in the United States: estradiol, testosterone, progesterone, trenbolone acetate, melengestrol acetate and zeranol."[5] Estradiol (a type of estrogen), testosterone and progesterone are hormones that are produced naturally in humans and livestock.

The rational behind the theory of saying it is safe to consume implanted meat is the fact that people are exposed to rather large quantities of these hormones anyway through their own body's daily production and naturally from various foods. The production of your body outweighs the small amount taken in addition from implanted meats. Therefore, it is believed that the additional amount of hormone ingested in eating beef from hormone implanted livestock is insignificant.[3] The conclusions the FDA reached was if consumers eat meat which contains 1 percent or less of the amount of hormones their own bodies produce, no effect could be expected.

For example, Estradiol is a natural estrogen found in many implant products.[1] A comparison of the intake from various foods and implanted beef is shown below:

Table 1. Estrogenic Activity of Several Common Foods[1]

Food	Estrogenic Activity (ng/500g food)
Soybean Oil	1,000,000
Cabbage	12,000
Wheat Germ	2,000
Peas	2,000
Eggs	17,500
Ice Cream	3,000
Beef from pregnant female	700
Beef from implanted cattle	11
Beef from non-implanted cattle	8

The effectiveness of natural estrogens is low so the amount of estradiol from implants would appear to pose no additional human safety risk. The synthetic estrogen, Zeranol is also an improved compound in implants. The dosage from implants can also be shown to be small.

The FDA ran extensive toxicological testing in animals to determine the safety and appropriate tissue residue tolerance levels of trenbolone acetate and zeranol before they were approved for use.[3] The tissue tolerance levels allowed by the FDA are 50 parts per billion for trenbolone acetate and 20 parts per billion for zeranol.

The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture conducts the National Residue Program for unacceptable levels. "No monitoring is done of naturally occurring hormones."[3] Monitoring for naturally occurring hormones is considered unnecessary by the FDA for two reasons. They believe the concentrations that are present are way below the level that would cause harm and second that the analysis method would not be able to distinguish between hormones naturally produced by the animal versus the implant. Hartman and Steinhart also state that it is

impossible to distinguish between endogenous and administered hormones.[6] Although, FSIS routinely monitors for synthetic hormones. FSIS also monitors for diethylstilbestrol (DES) a hormone which is no longer permitted in the United States.[3]

The Codex Committee on Residues of Veterinary Drugs in Foods, which is a committee of eleven scientific experts from seven countries, concluded that the use of naturally occurring hormones were unlikely to pose a hazard to human health and that with proper use neither would the synthetic ones.[3]

The purpose of this research project is to perform a pilot study and analyze samples of muscle tissue from beef for levels of approved and illegal steroids. Due to time constraints only a limited amount of samples may be analyzed. Time permitting approximately 20 samples can be analyzed for hormone levels. These samples will be chosen from local grocery stores and will include samples of products claiming to be hormone free. A sample from a private farm with no growth hormone use will also be evaluated and referred to as a blank. It is our goal to determine if there are significant levels of illegal or approved growth hormones in the tissue samples beyond that allowable according to guidelines established by the FDA.

There are several methods for detection of steroid hormones recommended by the EEC which include thin layer chromatography (TLC), liquid chromatography (LC), immunoassays, gas chromatography (GC), mass spectrometry (MS) and spectrometry.[6] A combination of those listed is advantageous. Immunoassays are suitable for screening of many samples for a small number of hormonally active substances with great sensitivity.[6] Gas chromatography coupled with mass spectrometry (GC-MS) is preferred if a limited number of samples need to be screened for multiple samples.[6]

The method developed by Verbeke uses TLC.[7] This advantage of this method that it is applicable to androgenic progestogenic and estrogenic with steroids or stilbene structures. The steps involved in this process are as follows: enzymatic hydrolysis of conjugated steroids, extraction with methanol, defatting step (liquid-liquid extraction with hexane), extraction with methylene chloride, isolation with a polystyrene column, separation of phenolic and natural steroids, further purification by passage through a celite-KOH column coupled to an Al_2O_3-column, derivitization and then GC-MS. The disadvantages of this method are the complicated extraction and clean-up procedure which takes 2.5 days and it requires more than 900 ml of solvent per sample including toxicologically problematic ones.

A method similar to the one which our research will follow is one established by P. Marchand et al.[7] This method uses lyphilization of the sample by freeze drying, liquid-liquid extration, deconjugation using *Helix pomatia*, followed by several different solid phase extractions to separate and purify the steroids.

The method we propose to follow is the method developed by Hartmann and Steinhart.[6] This method was chosen since it is a rapid and economical method which allows for the determination of anabolic and catabolic steroid hormones using gas chromatography-mass spectrometry (GC-MS). The sample is first homogenized using a rod homogenizer. This procedure replaces self-preparation columns with disposable solid-phase extraction cartridges (SPE). The use of these cartridges provides a reduction in time and solvent use. This method minimizes the use of time and chemicals. It also replaced ecologically harmful solvents with other suitable solvents. This method also deletes the enezymatic hydrolosis which some scientists recommend. The enzymatic

hydrolysis is normally performed by incubation of meat samples with β-glucuronidase-arylsulfatase from *Helix pomatia* overnight. Hartmann and Steinhart do not believe this step is necessary especially for the analysis of anabolic androgens, estrogens, and progestogens in muscle tissue. Hartman and Steinhart found that no significant hormone liberation was observed therefore the overnight incubation could be emitted saving more time which differs from P. Marchand et al, who states that a significant proportion does result in an increase of significant results for those steroids that are glucorono- or sulpho-conjugated and sometimes linked to glycosyl groups.[8] P Marchand et al, freeze-dries the sample because they believe that lyophilization of the sample results in an increased interaction between sample and solvent, a better extraction yield, and more efficient deconjugation.[8] It is possible to use the initial step of freeze drying versus rod homogenization which will also result in a decrease in solvent use and time.

Experimental Details:

The steps of the procedure as outline by Hartmann and Steinhart are as follows : (1) homogenization of meat sample (or lyophilization according to P. Marchand et al); (2) extraction/deproteinization; (3) delipidation; (4) separation of polar steroids from non-polar steroids; (5) separation of the non-polar steroids into neutral steroids and phenolic steroids; (6) purification of neutral steroids, phenolic steroids and polar steroids; (7) derivatization of steroids; and (8) analysis of steroid content using GC-MS.

Sample preparation will begin with 20-gram portions of ground beef placed in 70-ml methanol. This suspension will be placed in an ice bath and then 20 ml of water will be added. The suspension will then be homogenized using a rod homogeniser. This mixture will then be heated in a water bath at 60°C for 15 minutes then cooled to room temperature. The homogenate should then be centrifuged at 2000g for 10 minutes. To remove the fat an extraction can be performed on the supernatant with two portions of 20 ml hexane. The aqueous layer can be collected in a 250 ml round bottom flask. The methanol can than be evaporated by vacuum at 45°C. Once the remaining aqueous layer has cooled to room temperature solid phase extraction should be performed. The reader should note that the method of sample preparation will change if it is determined that lyophilization can be used in place of homogenization with the rod homogeniser.

Separation and clean up of the crude extract from above can be performed using a C_8-SPE cartridge. Conditioning of the cartridge can be performed with 4 ml methanol and 6 ml water. The remaining aqueous layer from above can be applied to the cartridge using a 20 ml reservoir with a weak vacuum. The flask and cartridge should be rinsed with two portions of 2-ml water and 2-ml methanol-water (40:60, v/v). The eluents should be collected in an 8-ml tapered glass tube. Fraction 1, the phenolic steroids, will be eluted with 2.5-ml methanol-water (60:40, v/v). Fraction 2, the non-polar steroids, will be eluted with 2.5 ml methanol. The solvents can be removed under nitrogen at 45°C.

Fraction 1 can be purified using a Si-SPE cartridge. The dry residue of fraction 1 should be dissolved in 2-ml of ethyl acetate (water saturated) with the aid of ultrasonication. The Si-SPE cartridge will be conditioned with 4-ml of ethyl acetate water saturated. The sample will be placed in the cartridge and filtered through with no vacuum. The tube and cartridge will be rinsed with two portions of 1-ml ethyl acetate

(water saturated). All eluent of the sample application and rinsing will be collected in a tapered 8-ml glass tube, and the solvent will be removed under nitrogen at 45°C.

Separation of fraction 2, non-polar steroids, can be performed using hexane/KOH. The dry residue of fraction 2 should be dissolved in 200 µl chloroform with the aid of ultrasonication, and 1.8-ml of hexane should be added. 2-mL of 0.25 M KOH aq. will be added, and the solution will be thoroughly mixed for one minute followed by centrifuge for two minutes. Separation can be increased by adding a few drops of ethanol. The lower phase, fraction 2b the phenol steroids, is then transferred to an 8-ml tapered glass tube containing 0.5 ml 1 M HCl. The pH of this layer should be neutral or weakly acidic.

The second layer from above, fraction 2a the neutral steroids, can be purified using a Si-SPE cartridge. The Si-SPE cartridge is preconditioned with 2x 2ml hexane-ethyl acetate (90:10v/v). Steroids are eluted with 2.5-ml hexane-ethyl acetate (25:75, v/v). Eluent is collected in an 8-ml tapered glass tube and the solvent is blown down under nitrogen at 45°C.

A re-extraction of the phenolic steroid, fraction 2b can be performed by agitation with 3 x 2-ml diethyl ether followed by centrifugation for about 1 min. The ether phases should be combined, and the solvent removed under nitrogen at 45°C. The dry residue left can then be dissolved in 2-ml ethyl acetate-methanol (80:20 v/v) with the aid of sonication. The sample should then be run through an NH_2-SPE cartridge previously conditioned with 4-ml ethyl acetate (water saturated) and 4-ml acetate methanol (80:20 v/v). The eluents from the sample application and the rinsing should be combined, and the solvent should be removed under nitrogen at 45°.

The following methanolic solution internal standards should be added to the purified extracts: 0.5µg/kg methyltestosterone, 0.25µg/kg estradiol-D_3, 1.0µg/kg medroxyprogesterone, 2.0µg/kg fludrocortisone, and the methanol should be evaporated under nitrogen.

Derivitization can be carried out in the 8 ml glass tubes by adding 40µl MSTFA-TMIS-DTE (1000:2:2, v/v/w). The tubes should then be sealed and heated at 60°C overnight to complete reaction.

Injections of the above extractions are now ready for injection into the GC-MS (1µl volume). The conditions for GC used helium as the carrier gas with a flow rate of 1.1 ml/min. The oven temperature ran from 130°C (1min) to 290°C at 12°C/min, 20 min isotherm. The injection temperature was 260°C.

The data from the experiment will be analyzed using conventional GC-MS analysis techniques. The quantitative values for residue levels will be compared to the legal limits set by the FDA. In addition to the comparison to legal limits, the residue levels for "normal" store-bought beef will be compared to those for beef marketed as growth hormone free. Future work in this area might be concerned with finding a better, more sensitive method, or perhaps evaluation of other animal food products. Future work may become more important, or perhaps obsolete, if conclusive scientific evidence is found concerning the effects on health of human consumption of hormone raised beef.

Project Needs:

	Amount	~Price ($)	Supplier
Methanol	2500 mL	18.10/L	Aldrich
Ethanol	1500 mL	21.15/L	Aldrich
Hexane	1500 mL	24.90/L	Aldrich
Ethyl Acetate	1000 mL	22.90/L	Aldrich
Diethyl Ether	1000 mL	32.90/L	Aldrich
Chloroform	5000 mL	25.20/L	Aldrich
1,4-dithioerythritol	1500 μL	17.70	Lancaster
HCl	500 mL	In Lab	
KOK	500 mL	In Lab	
N-methyl-N-trimethylsillytifluoroacetamide	1500 μL	26.20 / 5g	Lancaster
Trimethyliodosilane	1500 μL		
SPE cartridges (C_8, Si, NH_2)	(25, 50, 25)		
20 mL poplypropylene reservoirs			
Vacuum manifold			
Demineralized water	1500 mL		
8-mL glass tubes tapered for centrifuge	16		
Methyltestosterone	15 μL	8.30/1g	Sigma
Estradiol-D_3	10 μL	350/5mg	Cambridge Isotope Laboratories
Medroxyprogesterone	25 μL	74.30/g	
Fludrocortisone	50 μL		

References:

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(3) http://www.inform.umd.edu/EdRes/Topic/AgrEnv/ndd/safefood/HORMONES_AND_MEAT.html13/1/00 (accessed Feb 27, 2000)
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